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Association of malondialdehyde–acetaldehyde (MAA) adducted proteins with atherosclerotic-induced vascular inflammatory injury

Gary E. Hill [a,b,*], Jacqueline A. Miller [a], B. Timothy Baxter [c], Lynell W. Klassen [a], Michael J. Duryee [a], Dean J. Tuma [a,d], Geoffrey M. Thiele [a,d]

[a] *Department of Internal Medicine, University of Nebraska Medical Center, 600 S. 42nd Street, Omaha, NE 68198-4455, USA*
[b] *Department of Anesthesiology, University of Nebraska Medical Center, 600 S. 42nd Street, Omaha, NE 68198-4455, USA*
[c] *Department of Surgery, University of Nebraska Medical Center, 600 S. 42nd Street, Omaha, NE 68198-4455, USA*
[d] *The VA Alcohol Research Center, the Veterans Affairs Medical Center, Omaha, NE, USA*

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Abstract

Atherosclerosis is a vascular injury characterized by elevated tissue levels of tumor necrosis factor-α (TNF-α), increased expression of endothelial cell adhesion molecules, and vascular wall inflammatory cell infiltration. Foam cells are associated with atherosclerotic plaque material, and low density lipoprotein (LDL) is a lipid component of foam cells. Malondialdehyde (MDA) is an oxidative product of unsaturated fatty acids and is also present in atherosclerotic lesions. MDA-modified (adducted) proteins, including MDA-modified LDL, are present in atherosclerotic human vascular tissue. Acetaldehyde (AA) is the major metabolic product of ethanol oxidation. Both MDA and AA are highly reactive aldehydes and will combine with proteins to produce an antigenically distinct protein adduct, termed the MAA adduct. This study demonstrates that proteins modified in the presence of high concentrations of MDA can produce MAA-modified proteins in vitro. In addition, MAA adducted proteins are capable of inducing rat heart endothelial cell cultures (rHEC) to produce and release TNF-α, and cause rHEC upregulation of endothelial adhesion molecule expression, including ICAM-1. These adhesion molecules are required for circulating inflammatory cells to adhere to endothelium which allows inflammatory cell tissue infiltration. Additionally, MAA modified proteins were defected in human atherosclerotic aortic vascular tissue but not in normal aortic tissue. Since atherosclerosis is associated with an inflammatory vascular injury characterized by elevated tissue TNF-α concentrations and inflammatory cell infiltration, these data suggest that MAA-adducted proteins may be formed in atherosclerotic plaque material and may be involved in the inflammatory reaction that occurs in atherosclerosis. These data further suggest that previous studies demonstrating MDA modified protein in atherosclerotic plaque may in fact have MAA modified proteins associated with them. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atherosclerosis; Acetaldehyde; Malondialdehyde; Protein adducts; Inflammation

1. Introduction

Recent evidence suggests atherosclerosis is an inflammatory injury [1] as demonstrated by the upregulation and release of pro-inflammatory cytokines and the presence of CD4+ cells and macrophages in atherosclerotic plaques. Recent studies have demonstrated endothelial cell adhesion molecule surface expression is upregulated by the pro-inflammatory cytokines, and may serve as a site to facilitate inflammatory cell adhesion and infiltration [2]. This increased expression of endothelial cell adhesion molecules attracts circulating inflammatory cells which may play a prominent role in the atherogenic process [1]. The intercellular adhesion molecule-1 (ICAM-1), present on endothelial cell sur-

* Corresponding author. Tel.: +1-402-559-4081; fax: +1-402-559-7372; e-mail: ghill@unmc.edu.

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faces, can bind neutrophils and lymphocytes to the endothelial surface, and can be induced in vitro (increased surface expression) by tumor necrosis factor-α (TNF-α) [2]. Vascular cell adhesion molecule (VCAM) has kinetics similar to ICAM-1, is upregulated by the proinflammatory cytokines, and enables endothelial-lymphocyte adherence. In addition, major histocompatibility complex (MHC) molecules class I and II are receptors for T cell recognition and have been shown to be upregulated by pro-inflammatory cytokines [3,4]. The central issues associated with autoimmune reactions in atherosclerosis revolve around the nature of the antigens involved and the cellular interactions that must occur for the development of atherosclerosis. The inducing antigens have not been unequivocally identified; but, oxidized low density lipoprotein (LDL), viral antigens, and heat shock proteins have been shown to elicit immune responses [1].

Malondialdehyde (MDA), one of the major products of lipid peroxidation, is found in arterial walls associated with atherosclerotic plaques [5], and has been suggested to be another antigen associated with atherosclerosis. Lipid laden macrophages, or foam cells, are consistently present in atherosclerotic arterial wall [1]. Oxidized LDLs are a major source of foam cell lipid, while MDA-modified LDL is taken up by these cells by a specific scavenger mechanism and is thought to be involved in the inflammatory vascular injury described with atherosclerosis [6]. Specific monoclonal antibodies to MDA-modified LDL have demonstrated that this aldehyde–protein adduct is present in atherosclerotic plaques [7]. Malondialdehyde is an unstable and reactive compound that may bind to lysine residues [8,9]. Previous studies by Tuma et al have demonstrated that MDA and acetaldehyde (AA) react together with endogenous proteins to generate an antigenically distinct protein adduct, termed the MAA adduct [10]. Recently, it has been demonstrated that intrahepatic MAA adduct formation may be the consequence of the metabolism of ingested ethanol which results in AA generation in combination with hepatic lipid peroxidation which produces MAA [10]. It is relevant to note that recent evidence has demonstrated that heavy alcohol consumption (five to six drinks daily) results in accelerated atherogenesis [11].

In this report, we present evidence that MAA adducted proteins induce rat heart endothelial cell TNF-a release and subsequent upregulation (increased expression) of endothelial cell surface adhesion molecules. In addition, MAA adducted proteins were detected in human atherosclerotic aortic walls but not in normal nonatherosclerotic aortic walls. Therefore, these data suggest that the induction of adhesion molecules and proinflammatory cytokines in response to MAA adducted proteins may play a role in the pathogenesis of human atherosclerosis.

2. Materials and methods

2.1. Modification of proteins

MDA was obtained as the sodium salt by treatment of tetramethoxypropane (Aldrich, Milwaukee, WI) with NaOH as previously described [10]. Bovine serum albumin (BSA) was modified with MDA and/or AA. Briefly, MDA modified BSA (BSA–MDA) was produced by incubating BSA with MDA (1, 5, 10, 20 and 50 mmol/l) in phosphate buffer (0.1 mol/l, pH 7.4) at 37°C in polypropylene vessels. The reactions were performed under nitrogen gas in the dark for 24 or 72 h. Samples were dialyzed for 24 h at 4°C.

Production of the malondialdehyde–acetaldehyde adduct by BSA modification: BSA–MAA was prepared as described by Tuma et al. [10]. Briefly, BSA (Sigma, St. Louis, MO) was modified with 1 mM malondialdehyde and 1 mM [^{14}C]-acetaldehyde (New England Nuclear, Boston, MA) by incubating at 37°C for 72 h. Dialysis and fluorescence measurements were performed as previously described [10]. Postincubation, free and unstable-bound MDA or AA were separated by exhaustive dialysis against phosphate buffer for 24 h at 4°C. Radioactivity was then measured in the retentate and represented stably bound MAA. Fluorescence measurements were obtained on postdialysis samples using a Perkin Elmer (Norwalk, CT) LS-5B spectrophotofluorometer attached to a Perkin Elmer GP-100 graphics printer as previously described [10]. Protein concentrations of the retentate was measured as described by Bradford [12].

2.2. Indirect ELISA to detect the amount of antibody bound to MDA-modified proteins

BSA modified with MDA at 0, 1, 5, 10, 20 and 50 mM for 24 and 72 h was coated overnight onto ELISA plates (Immulon IV, Dynatech, Chantilly, VA) with the MDAmodified proteins at 2 μg/well. A characterized polyclonal rabbit anti-MAA antibody [10] was incubated in the assay for 45 min at 37°C. The polyclonal rabbit anti-MAA antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (H&L)(Jackson Immunologicals, West Grove, PA). After adding the substrate, optical density measurements of the microtiter plates were read with a Dynatech MR 7000 plate reader at 405 nm.

2.3. Indirect competitive ELISA to detect MAA adduct formation following MDA modification

BSA was modified with MDA at 0, 1, 5, 10, 20 and 50 mM for 24 or 72 h. The amount of MAA-modification through MDA was determined by an indirect competitive ELISA developed to quantify the amount

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of MAA-modification of protein [10]. Briefly, ELISA plates were coated with 2 μg/well of BSA, BSA–MAA, and BSA–MDA (1, 5, 10, 20 or 50 mM modified) and incubated overnight. Polyclonal antibody to MAA (at one half maximum binding) was incubated with varying concentrations of hexyl MAA (a known inhibitor) [13] overnite at 4°C, added to duplicate wells of the coated plates and incubated for 45 min at 37°C. After washing, alkaline phosphataseconjugated goat anti-rabbit IgG (H&L) (Jackson Immunologicals, West Grove, PA) was added, and procedures as described for the indirect ELISA were used to obtain optical density measurements [10]. Results for each inhibitor concentration were expressed as percent inhibition, which was calculated using the formula developed by Roberts et al. [14]:

$$\%\text{Inhibition} = \frac{(OD_{max} - BKG) - (OD_{sample} - BKG)}{(OD_{max} - BKG)} \times 100$$

where OD_{max} is the OD in the absence of inhibitor, BKG is the OD from nonspecific absorption of assay reagents and OD_{sample} is the OD for a given concentration of standard.

2.4. Isolation and culture of heart endothelial cells

Male Wistar rats were purchased from Charles River (Wilmington, MA), anesthestized by intraperitoneal phenobarbital (100 mg/kg) and the intact beating heart was removed. After mincing and dispase digestion, heart endothelial cells (HECs) were isolated and grown to confluency as described by others [3]. HECs were separated by centrifugation at $400 \times g$ for 10 min followed by three washes with M199-F12 containing 10% fetal calf serum. The cells collected were > 90% HECs, verified by staining with mouse anti-rat RECA-1 (Harlan Bioproducts for Science, Indianapolis, IN) and mouse anti-factor VIII-von Willebrand's factor (Cedar Lane Laboratories, Hornby, Ont., Canada). Cells were seeded into 24-well tissue culture plates (Becton-Dickinson Labware, Franklin Lakes, NJ) containing fibronectin (Sigma, St. Louis, MO) coated at 20 μg/well on glass coverslips and grown to confluency at 37°C over 48–72 h. The cells were washed on the day of the experiment with M199-F12 without serum and allowed to incubate for 2 h before assaying. HECs were exposed to BSA (negative control), lipopolysaccharide (LPS, 10 ng/ml) [15] (Sigma, St. Louis, MO) (positive control), or 1 or 25 μg of BSA–MAA, 1 or 20 mM BSA–MDA, or hexyl MAA for 1, 3 or 24 h.

2.5. TNF-alpha ELISA

Quantification of TNF-a levels from the supernatants of HEC cultured cells was performed by Factor-Test-X™ rat TNF-α ELISA kit (Genzyme Diagnostics, Cambridge, MA), which employs a multiple antibody sandwich principle. A 96-well microtiter plate precoated with monoclonal anti-TNF-α antibody was used to capture rat TNF-α from standards and test samples. After washing, a peroxidase conjugated polyclonal anti TNF-α antibody bound to captured rat TNF-α. After incubation, the plate was washed again to remove unbound antibody and substrate solution added to initiate color change. The absorbance was measured at 450 nm and was proportional to the concentration of rat TNF-α in the standard or sample. The standard curve was obtained by plotting the concentrations of rat TNF-α standard versus absorbance. The sample concentrations of rat TNF-αwere determined by extrapolation using the standard curve. Final concentrations of TNF-α were expressed in pg/ml.

2.6. Percentage of cell necrosis determinations

The percentage of cell necrosis (death) of the HECs during exposure to BSA–MAA was determined by an enzyme (lactic acid dehydrogenase, LDH) release assay of the HEC supernatant as described by Korzeniewski and Callewaert [16]. In brief, following stimulation of the HECs with 1, 5, 10 and 25 μg BSA–MAA or media only (control) for 3 and 24 h, the HECs were centrifuged ($200 \times g$, 10 min). A 100-μl aliquot of the HEC supernatant was transferred to the corresponding wells of flat-bottomed microtiter plates. Subsequently, 100 μl of a freshly prepared lactic acid dehydrogenase substrate mixture (5.4×10^{-2} M lactate (Acros Organics, NJ), 6.6×10^{-4} M 2*p*-iodophenyl-3*p*-nitrophenyl tetrazolium chloride (Acros), 2.8×10^{-4} M phenazine methosulfate (Acros) and 1.3×10^{-3} M nicotineamide nucleotide NAD in 0.2 M Tris buffer, pH 8.2 (Sigma)) was added to each well. The plates were incubated light-protected at room temperature for 10 min and the reaction stopped by the addition of 50 μl 1 M HCl/well. A microtiter plate reader (MR 700, Dynatech Laboratories, Chantilly, VA) was used to monitor the resultant light absorbance at 490 and a 630 nm reference. The LDH activity, expressed as change in absorbance/min, was calculated with Biolinx 2.21 software (Dynatech) on an IBM compatible computer. Percentage necrosis of the HECs was determined by the following formula:

$$\%\text{Necrosis} = (E - S)/(M - S) \times 100,$$

where E is the optical density (OD) of the experimentally induced release of LDH activity from the HECs incubated in the presence of the various concentrations

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of BSA-MAA, S is the spontaneous release of LDH activity (OD) from HECs incubated with media only, and M is the maximal release of LDH activity (OD) determined by total HEC necrosis induced by exposure to 10% Triton X-100 (Fisher Scientific, Fair Lawn, NJ).

2.7. Immunohistochemistry

Following harvest of HEC culture supernatants for the TNF-α assay, coverslips from the 24-well plates were washed with phosphate buffered saline (PBS) and fixed with ice cold acetone-methanol (50:50 v/v). The cells were stained by standard techniques for the following cell surface markers: MHC class I, MHC class II, ICAM-1 and VCAM (Pharmingen, Palo Alto, CA). The coverslips were removed following staining, inverted and placed on microscopic slides with mounting media [17]. A graded scoring system was performed and assessed by two independent observers. An isotype control and FITC labeled second antibody were used for determining background staining. All slides were viewed on a Nikon LaboPhot microscope (Sheerin Scientific, Kansas City, MO) and filmed with Kodak Ektachrome 320T film.

2.8. Human aortic tissue

Following Institutional Review Board approval and informed patient or family consent, sections of the infrarenal abdominal aorta was removed from non-alcoholic humans undergoing elective aortic revascularization surgery (sections were taken through plaque material) or from organ donors with no indications of atherosclerosis of the aorta. The aortic sections were fixed in OCT compound, frozen at −70°C, and mounted on standard microscopic glass slides. For staining, the slides were thawed, fixed with a solution of cold acetone–methanol (50:50), blocked with 1% powdered milk, and incubated with polyclonal rabbit anti-MAA antibody, or rabbit IgG (Sigma) at equal concentrations to the polyclonal antibody (negative control) for 30 min. The slides were washed with PBS and an FITC-labeled goat anti-rabbit IgG antibody (Jackson, West Grove, PA) was added to the aortic strips for 30 min. After a final wash, the slides were dried and mounted using a quenching solution [17]. In some cases, antibody was incubated overnight at 4°C with a synthetic compound (hexyl–MAA) that has been shown to inhibit antibody binding. These studies were performed to demonstrate the specificity of the antibody used and showing that non-specific binding was not observed. Photomicrographs of the resulting slides were taken with Kodak Ektachrome 320T film with a Nikon LaboPhot microscope (Sheerin Scientific, Kansas City, MO). In addition, photomicrographs were taken of hematoxylin/eosin (H/E) preparations of the same tissue sections of atherosclerotic infrarenal aorta used for the demonstration of the presence of MAA-adducted protein.

3. Statistical analysis

All results are reported as means ± S.E.M. or S.D. Analysis of variance (ANOVA) or the Student's t-test were used to determine significance. P values of 0.05 or less defined significance [18].

4. Results

4.1. MDA modification of BSA

Other investigators have shown that MDA can modify proteins and elicit antibody responses [5–7]. It has recently been reported that under certain conditions, high concentrations of MDA may form acetaldehyde [18,19]. Since the (2:1) MAA-product (two molecules of MDA and one of acetaldehyde [13]) may form when there is excess MDA when compared to AA [10], it is possible that the 2:1 product reported by Tuma et al. [10] may be produced. As can be seen in Fig. 1A and B, an antibody previously reported to be specific for only the MAA adduct reacted with BSA–MAA (positive control) and MDA at 5, 10, 20 and 50 mM (24 and 72 h assay). As expected, the affinity purified rabbit anti-MAA antibody did not react with BSA (negative control) or the 1 mM MDA. Therefore, these data show that high concentrations of MDA (>1 mM) may result in the production of MAA products. While the specificity of the affinity purified rabbit anti-MAA antibody has been defined to react predominantly with the 2:1 (MDA:AA) fluorescent product [10,13], it was possible that at higher concentrations of MDA, it would react with another untested product. Therefore, inhibition assays using hexyl MAA (a synthetically prepared analog of the 2:1 product; courtesy of Mark Kearly, Ph.D., [13]) as the inhibitor were initiated. As shown in Table 1, the amount of adduction levered off at about 20 mM MDA as evidenced by the observation that no differences between 20 and 50 mM MDA was seen. Therefore, using 20 mM MDA modified BSA as the coating antigen, it was shown that the affinity purified rabbit anti-MAA antibody could be totally inhibited with approximately 5000 pg/ml of hexyl–MAA at 24 and 72 h (Fig. 2A and B). This compared favorably to high levels ($>70\%$) of inhibition, beginning with low (10 pg/ml) concentrations of the inhibitor (hexyl–MAA), of the binding of the affinity purified rabbit anti-MAA antibody to BSA–MAA (Fig. 2B). These data suggest that MAA adducts can be formed on proteins incubated with MDA at high concentrations (>2 mM).

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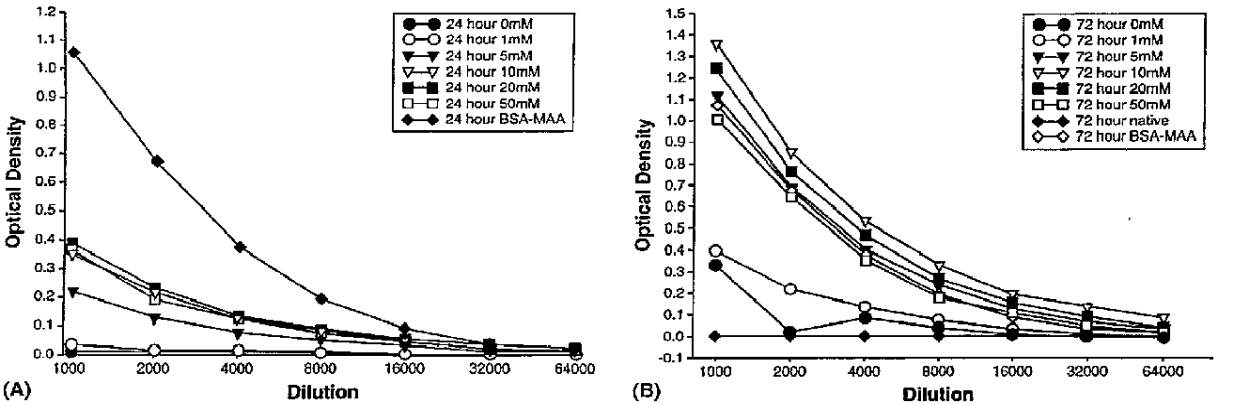

Fig. 1. Affinity purified rabbit anti-MAA antibody specificity for BSA only, BSA–MAA, and MDA–BSA (20 and 50 mM) modified proteins for 24-h (A) and 72-h (B) assays. BSA was modified with 0, 1, 5, 10, 20 and 50 mM MDA or 1 mM MDA and 1 mM AA for 24 h at 37°C (for MAA production). Native (unmodified) BSA (0 mM) and each of the modified BSAs were coated onto 96-well plates and exposed to the rabbit anti-MAA antibody. Optical density was read at 410 nm on a Dynatech MR 7000 plate reader and plotted against the dilution of the rabbit anti-MAA antibody.

4.2. In vitro heart endothelial cell TNF-α release

Studies have shown that endothelial cells from heart tissues will respond to MDA modified proteins. In order to determine whether MAA modified proteins induce an inflammatory response by heart endothelial cells, isolated cells were stimulated with media, BSA, BSA–MAA, hexyl–MAA, BSA–MDA, or LPS. The heart endothelial cell cultures stimulated with BSA–MAA, 1 or 25 μg/well, hexyl–MAA, 25 μg/well, 1 or 20 mM BSA–MDA, or LPS (10 ng/well) (positive control [15]) demonstrated significantly ($P < 0.05$) increased concentrations of TNF-α in the supernatant at all time periods when compared to control (media only) and BSA stimulated cells. The TNF-α concentrations in the wells stimulated with hexyl–MAA was significantly ($P < 0.05$) greater than the wells stimulated with BSA–MAA, BSA–MDA, and LPS. The heart endothelial cell culture supernatants stimulated with no antigen or BSA only demonstrated minimal TNF-α release (Fig. 3).

During the completion of these studies, it was observed that incubation with MAA-adducted proteins resulted in morphological changes of HECs. Both concentrations of BSA–MAA (1 and 25 μg) resulted in distinct morphological changes as compared to BSA alone. These changes included; rounding up of the cells, increased granularity, and detachment from the matrix. In order to determine if the MAA-adducted proteins were causing cell necrosis, the percentage of cell necrosis of the HEC was determined by the resultant concentration of supernatant LDH release [16] following stimulation with 1, 5, 10 or 25 μg BSA–MAA at 3 and 24 h. A significant increase in the percentage of HEC necrosis was found following stimulation with 10 and 25 μg BSA–MAA at the 3 and 24 h time periods when compared to control (media only) at the same time periods (Fig. 4).

4.3. Immunohistochemistry for adhesion molecules

Since TNF-α was released and the morphology of the cells changed so drastically in response to MAA, it was thought that adhesion molecule expression may also be altered. HECs exposed to nothing, BSA or BSA–MAA (1 μg/well) were analyzed for ICAM-1,VCAM, MHC class I and II antigen expression after a 24-h stimulation period (Table 2). MHC class I was used as a positive control since all cells express class I and there was only a modest increase in the amount expressed following exposure to BSA–MAA (2+ to 3+). In contrast, ICAM-1, MHC class II and VCAM were greatly increased when stimulated with BSA–MAA (1+ to 3+, ± to 3+, and − to 3+, respectively) (Table 2). In all cases, the isotype controls stained minimally.

4.4. Human aortic tissue

Since all of the changes associated with HECs were performed in the rat model, questions arose as to the significance of MAA in human atherosclerotic lesions. Human aortic tissue from known atherosclerotic aorta stained positively for MAA-adducted protein throughout all layers of the aortic wall when aortic tissue was exposed to the polyclonal rabbit anti-MAA antibody (1:1000) (Fig. 5A). When the polyclonal rabbit anti-MAA antibody was cultured overnight at 4°C with hexyl–MAA prior to aortic tissue exposure, the atherosclerotic aortic tissue stained sparingly (Fig. 5B).

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Table 1
The amount of fluorescent MAA/mg of protein at days 1 and 3

MDA	Day 1		Day 3	
	nmoles Fluorescence/mg protein	F/P (mol/mol)	nmoles Fluorescence/mg protein	F/P (mol/mol)
0	0	0	0	0
1	0.028	0.0019	0.037	0.0025
5	0.117	0.0080	0.222	0.0153
10	0.233	0.0160	0.422	0.0290
20	0.401	0.0276	0.715	0.0492
50	0.477	0.0328	0.639	0.0440

Known atherosclerotic aortic wall stained lightly with the goat anti-rabbit IgG FITC labeled monoclonal antibody (for background staining determination) (Fig. 5C). Normal aortic wall stained minimally for MAA-adducted protein throughout all layers of the aortic wall when aortic tissue was exposed to the polyclonal rabbit anti-MAA antibody (1:1000) and FITC labeled second antibody (Fig. 6A). Normal aortic wall stained minimally when exposed to the goat anti-rabbit IgG FITC labeled monoclonal antibody (for background staining determination) (Fig. 6B). Hematoxylin/eosin staining of the same atherosclerotic aortic tissue used for the demonstration of MAA-adducted protein demonstrated inflammatory cell infiltration (Fig. 7A and B).

5. Discussion

Atherosclerosis is the result of the interaction of multiple diverse factors such as lipid metabolism, blood coagulation, hemodynamic stress, behavioral risk and the proinflammatory cytokines. There are two main hypotheses that could explain the initiation of atherosclerosis: (1) modification of LDL, and (2) an inappropriate immune response resulting in vascular injury [1]. The central issues relating to possible autoimmune reactions in atherosclerosis center around the nature of the antigens involved and the cellular interactions that are required to initiate relevant effector functions. Antigens that may play a role in this disease have yet to be definitely identified, but MDA-oxidized LDL adducts have been implicated in eliciting immune responses [20]. Additionally, proinflammatory cytokines and upregulation of adhesion molecule expression have been proposed to play major roles in the development of atherosclerosis [1].

MDA-modified protein is present in human arterial vascular tissue and plasma of humans with known atherosclerosis [21] and is present in macrophage-derived foam cells in atherosclerotic aorta [6,20]. This is probably a result of the oxidative decomposition of unsaturated fatty acids that are a component of plaque material found in the atherosclerotic lesion [20]. Malondialdehyde has been shown to form many different products when incubated with proteins. One of these structures was a cyclic structure that results from the spontaneous breakdown of MDA to acetaldehyde [9]. This happens at only high concentrations of MDA and is a minor product [9]. The structure of this product is similar to that proposed by Tuma et al. [10], that has been shown to form following chronic ethanol consumption and has been termed MAA. In studies performed in this laboratory, it has been observed that MAA-adducted proteins can be bound and degraded by peritoneal macrophages and liver endothelial cells. Recently, cytokines (TNF-α, MCP-1, and MIP-2) and adhesion molecules (ICAM-1, VCAM-1) have been shown to be upregulated in liver endothelial cells in response to MAA-adducted proteins (Dr Thiele, unpublished data). Therefore, it was the purpose of this study to determine if MAA-adducted proteins are present in atherosclerotic lesions and if they potentially are involved in the development and/or progression of the inflammatory process observed in atherosclerosis.

Using a well-characterized antibody that has been shown to only recognize MAA-adducted proteins [10], it was possible to demonstrate that higher than physiologic concentrations of MDA will form this same product. Inhibition assays (with hexylamine) demonstrated that there was no other unidentified MDA product on these adducted proteins since the interaction was completely inhibited. It is important to note that the formation of MAA from MDA alone is dependent on a higher than physiologic concentration of MDA (1 mM). This raises the question of whether other investigators are actually detecting MDA in atherosclerotic lesions [5,7,22,20,21] since most antibodies have been prepared using 10–50 mM MDA modified proteins as the immunogen. Since these antibodies are being tested against only the immunogen, the investigators have assumed that their antibodies reacted with only MDA. Additionally, they did not perform inhibition assays with known structures to better determine their antibody specificity. The MAA product has been shown to form at physiologic concentrations of both MDA and

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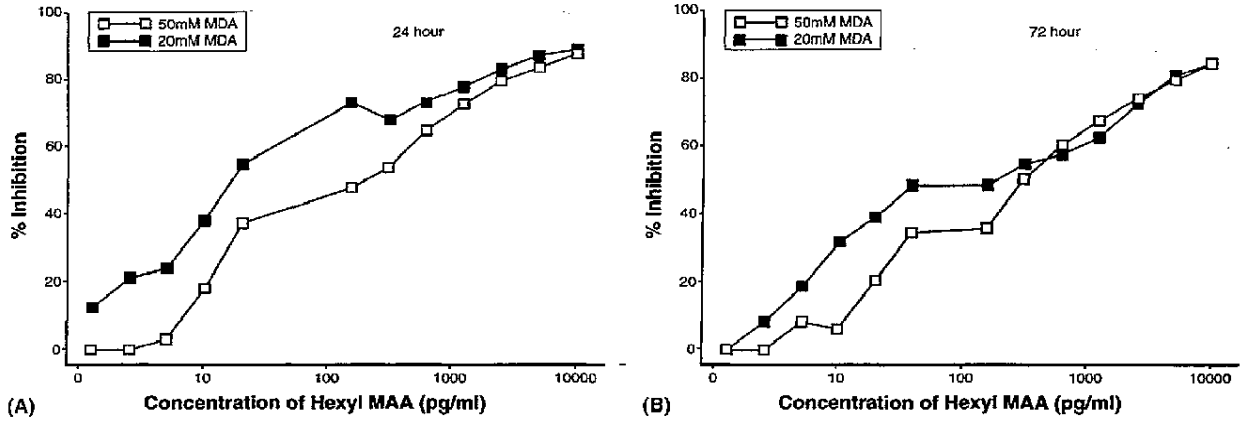

Fig. 2. (A) 24-h and (B) 72-h hexyl–MAA inhibition assay demonstrating the resulting inhibition curves, using hexyl–MAA as the inhibitor, for 20 and 50 mM malondialdehyde modified BSA.

AA (< 1mM) that would be achieved following chronic ethanol consumption [10,13].

In early atherogenesis, monocytes adhere to intact endothelium, thereby gaining access to the intima [1]. In studies reported in this manuscript, exposure of rat heart endothelial cells to MAA–BSA resulted in a pronounced morphological change in the endothelial cells and, more importantly, the upregulation of a number of adhesion molecules. These data would be consistent with the infiltration of more monocytes followed by CD4+ and CD8+ T cells into the area of inflammation [23]. The ability of endothelial cells to become a matrix for binding and extravasation of other inflammatory cells is reasonable in relationship to the results observed.

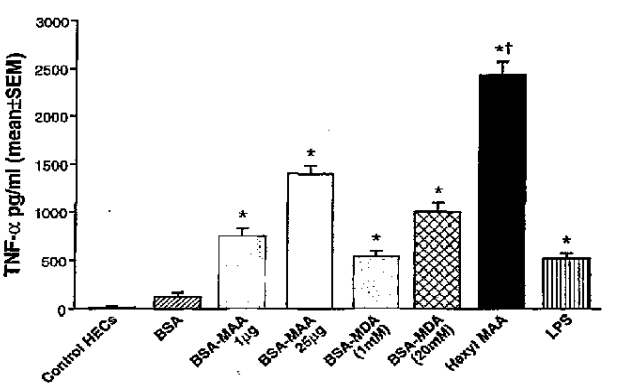

Fig. 3. Rat heart endothelial cell culture supernatant tumor necrosis factor-α concentrations (pg/ml) following stimulation for 3 h with no antigen (control), BSA only, or 1 or 25 µg/well of BSA–MAA, 1 or 20 mM BSA–MDA, or 25 µg/well of hexyl–MAA. Lipopolysaccharide, (LPS) (10 ng/well) was used as a positive control. * $P < 0.05$, when compared to no antigen and BSA only. + $P < 0.05$, when compared to BSA–MAA, BSA–MDA, and LPS ($n = 6$, each condition).

Although there is little evidence for cytotoxic reactions within plaques, a number of proinflammatory cytokines (IL-1, IL-6, TNF-α, and IFN-γ) are secreted into the plaque [1,24]. It has been suggested that these cytokines are secreted by one or more of the following cell types; macrophages, T cells, smooth muscle cells, and endothelium [25]. The secretion of TNF-α by heart endothelial cells following stimulation with MAA-adducted proteins is consistent with the observations outlined above. Preliminary studies using liver endothelial cells have shown that MCP-1 and MIP-2 are released along with TNF-a following stimulation with BSA-MAA (Dr Thiele, unpublished data). It is possible that the heart endothelial cells may secrete similar cytokine patterns that could result in the initiation and/or progression of atherosclerosis [1]. The mechanism of cell death (necrosis) found with the higher concentrations of BSA–MAA reported in Fig. 4 remains undefined. Tumor necrosis factor-α may induce cell death [26], therefore the significant increase in the percentage of HEC necrosis following stimulation with 10 and 25 µg of BSA–MAA may be due to the direct toxicity of BSA–MAA or indirectly due to the BSA–MAA-in-

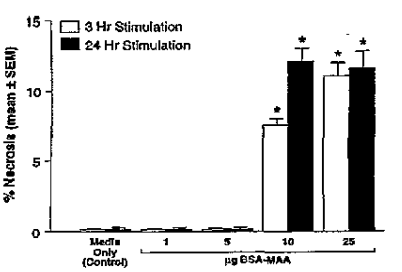

Fig. 4. Percentage of rat heart endothelial cell necrosis, determined by LDH release (see Section 2), for media only (confrol), or 1,5,10, and 25 p9 BSA–MAA stimulation for 3 and 24 h. * $P < 0.05$, when compared to control for the same time period ($n = 6$, each condition).

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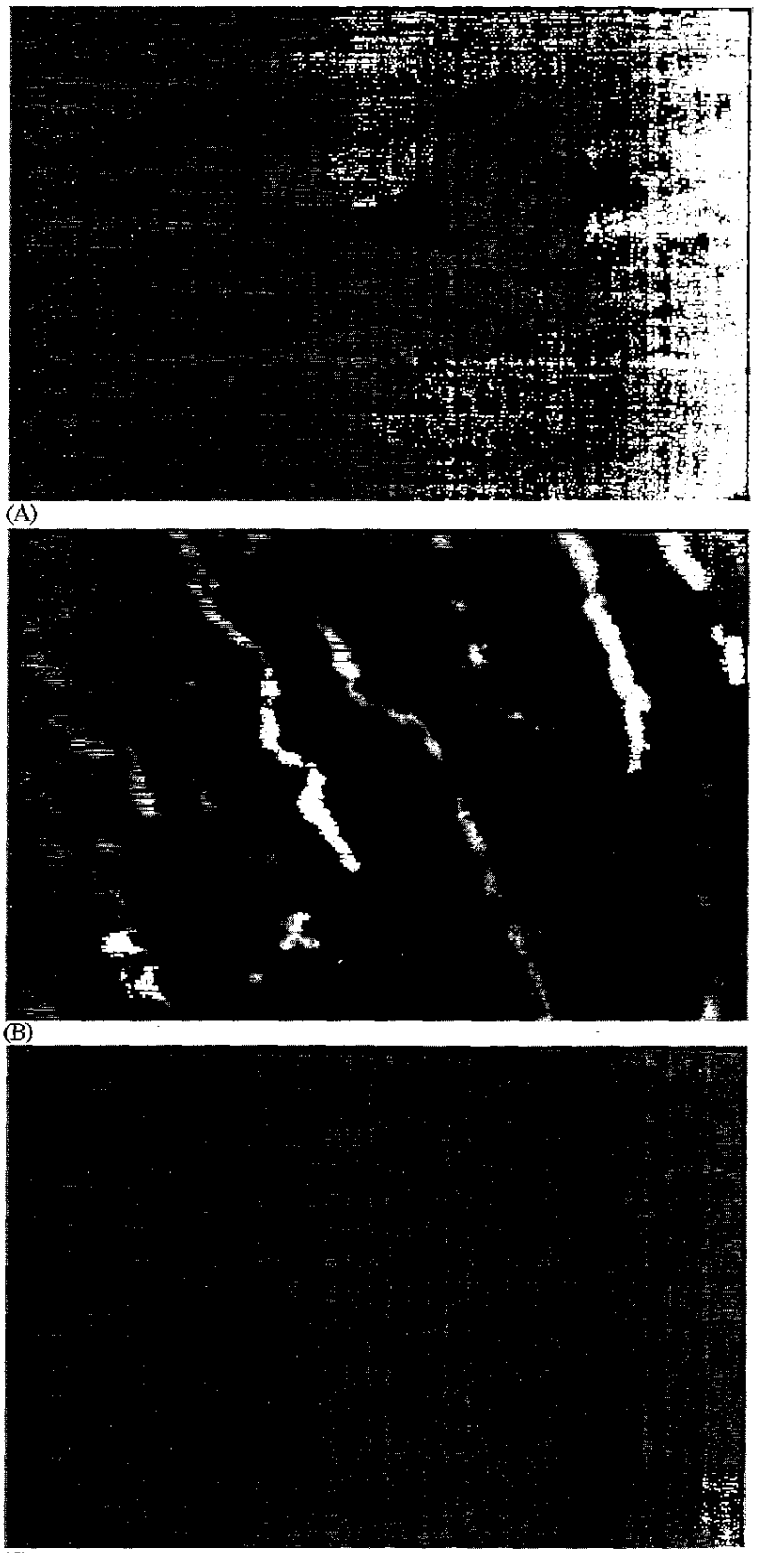

Fig. 5

Fig. 5. Immunohistochemistry of human atherosclerotic aortic tissue for (A) MAA-adducted protein, (B) the polyclonal anti-MAA antibody was inhibited by prior culture with an inhibitor (hexyl–MAA), and (C) non-specific background staining with anti-IgG FITC labeled monoclonal antibody. Magnification of 62.5 ×.

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Table 2
Graded scoring system for MHC class 1 antigen, MHC class II antigen, ICAM-1 and VCAM of rat heart endothelial cell surface adhesion molecule expression following a 24-h stimulation with bovine serum albumin (BSA) or 1 μg/well of BSA–MAA

	Class I		Class II		ICAM-1		VCAM	
	BSA	BSA–MAA	BSA	BSA–MAA	BSA	BSA–MAA	BSA	BSA–MAA
HECs	++	+++	+	+++	±	+++	–	+++

duced TNF-α release. Additional studies will be required to further define this issue.

There is strong evidence that MDA-modified proteins are detected in atherosclerotic tissue [5,7,20,21]. In studies reported in this manuscript, it was shown by a highly specific rabbit anti-MAA antibody that MAA-modified proteins are present in atherosclerotic aortic tissue. Additionally, using hexyl–MAA (a known inhibitor for this antibody) [10], it was possible to abolish this activity, demonstrating that this reaction was not due to cross-reactive epitopes. With the reports that high concentrations of MDA can form this MAA product [8,9,19,22] in addition to the demonstration of anti-MAA antibody activity to these modified proteins, it is possible that some of the anti-MDA antibody activity reported by other investigators in atherosclero-

(A)

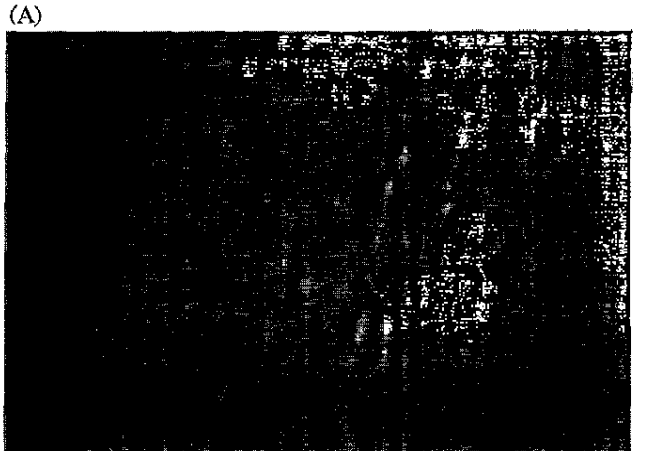
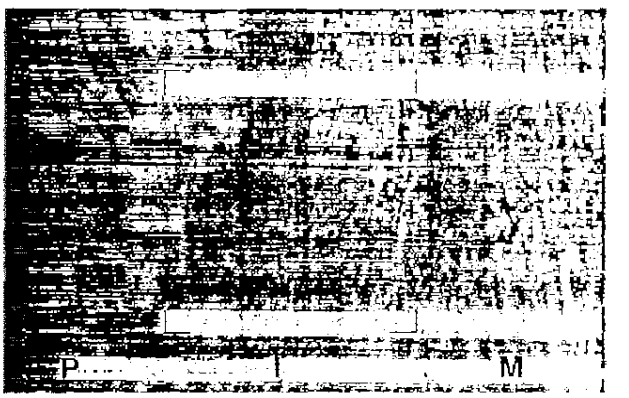
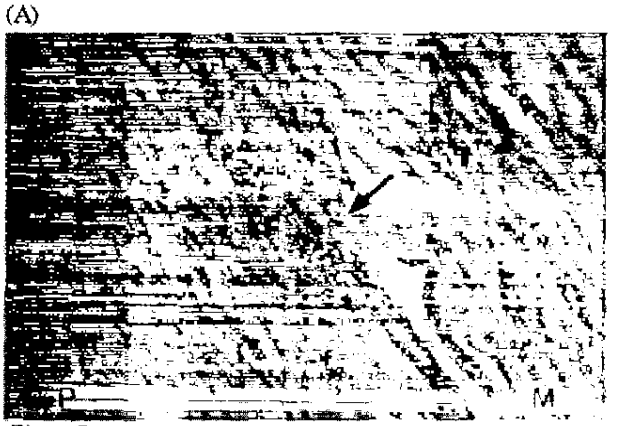

(B)

Fig. 6



(A)



(B)

Fig.7

Fig. 6. Immunohistochemistry of normal human aortic tissue for (A) MAA-adducted protein, and (B) non-specific background staining with anti-IgG FITC labeled monoclonal antibody. Magnification of 62.5 ×.

Fig. 7. Hematoxylin/eosin (H/E) preparation of human atherosclerotic aortic tissue demonstrating plaque (P), intima (I), and media (M). (A) Brackets outline area used for the immunohistochemistry for MAA-adducted protein. Magnification of 10 ×. (B) H/E preparation of the same tissue demonstrating inflammatory cell infiltration (indicated by arrow). Magnification 20 ×.

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sis [7] is due to the presence of MAA adducts. Consistent with these findings is the observation that heavy alcohol consumption (five to six drinks per day) accelerates human atherosclerotic heart disease [11]. The mechanism for this alcohol-induced accelerated atherogenesis is understandable since ingested alcohol is metabolized to AA and since MDA is already present secondary to lipid peroxidation in the atherosclerotic plaque, therefore, the formation of MAA would be enhanced [10].

In conclusion, the current study demonstrated that MAA-adducted proteins can induce heart endothelial cells to produce proinflammatory cytokines (TNF-α) and upregulate endothelial adhesion molecules. While not demonstrated in this study, it may be possible that these endothelial ceils could also present antigen within the context of MHC class I or II expression and potentially explain the presence of CD4+ and CD8+ T cells in atherosclerotic plaque [23]. Additionally, if MCP-1 and MIP-2 are produced by endothelial cells that have bound MAA-adducted proteins, then macrophages and neutrophils would be activated and attracted into an inflammatory site. Therefore, these data strongly suggest a role for endothelial cells and MAA-adducted proteins in the development and/or progression of atherosclerosis.

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